université PARIS-SACLAY | FACULTÉ DE PHARMACIE

Microbial production of therapeutic interest molecules

Master 2 Microbiology and Biological engineering

Teaching Unit "Medico- Pharmaceutical Applications of microbial biodiversity"

Inserm
Institut national
de la santé et de la recherche médicale

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Agenda

- Introduction: Health and Biotechnologies
- Production process for therapeutic bioproducts: USP/DSP
- Example for Antibiotics production
- Example for Recombinant Proteins

Introduction: Biotechnology Concept

Pharmaceutical biotechnolgy: process using mirobial factories, plants or animals for the production of pharmaceutical products

Bioproduction is the production of biologics-based therapeutic drugs

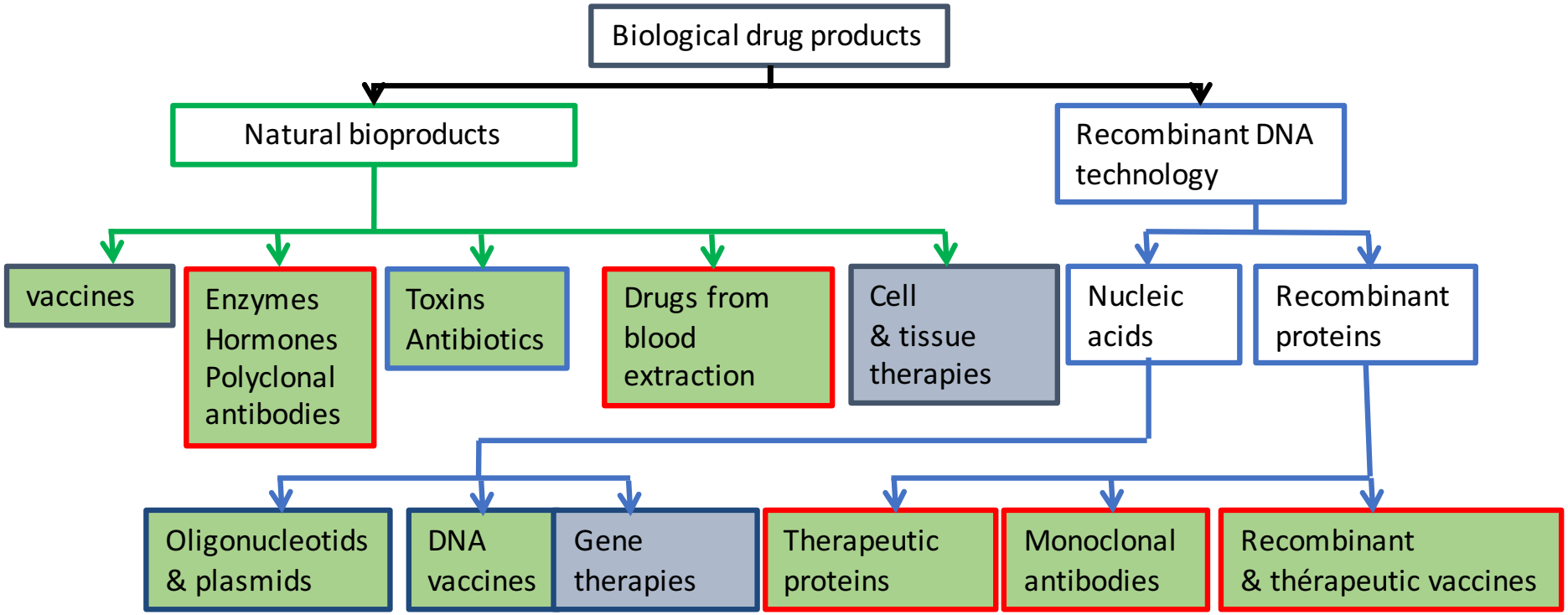
Biodrug:

- Drug substance produced by or extratcted from a **biological source**
- Well characterized & described **manufacturing and purification process**
- **Characterization** and **quality** assessment need a **combination** of physical, chemical and biological assays

EU definition of Biologic (Directive 2001/83/EC as amended, Annex 1 Active substance 3.2.1.1.b)

Biodrug classification

The CONCEPTION and the PRODUCTION are a biotechnological process



Industrial Production

Non industrial production = Innovative medicine

Protéins

Biogrugs and micro-organisms

- **Proteins:**
  - Recombinant: hormones, antibodies and derivates, cytokines…
    - Bacteria, yeast, insect cells, mammal cells
  - Natural production: enzymes, hormones, antibodies, albumin, collagen…
    - Bacteria, yeats, fungi, mammal cells
- **Nucleic acids**
  - DNA vaccines: bacteria
  - Plasmids, oligonucleotides: bacteria
  - Viral vectors: viruses (gene therapy)
- **Antibiotics:** fungi, bacteria
- **Lipids, polysaccharids, organic complex molecules**: Yeast, bacteria
- **Vaccines**
  - Microbial: virus, bacteria
  - Recombinants: bacteria, yeast, mammal cells, insect cells

The production process: an overview

Bioproducts need complex industrial biological mastered process

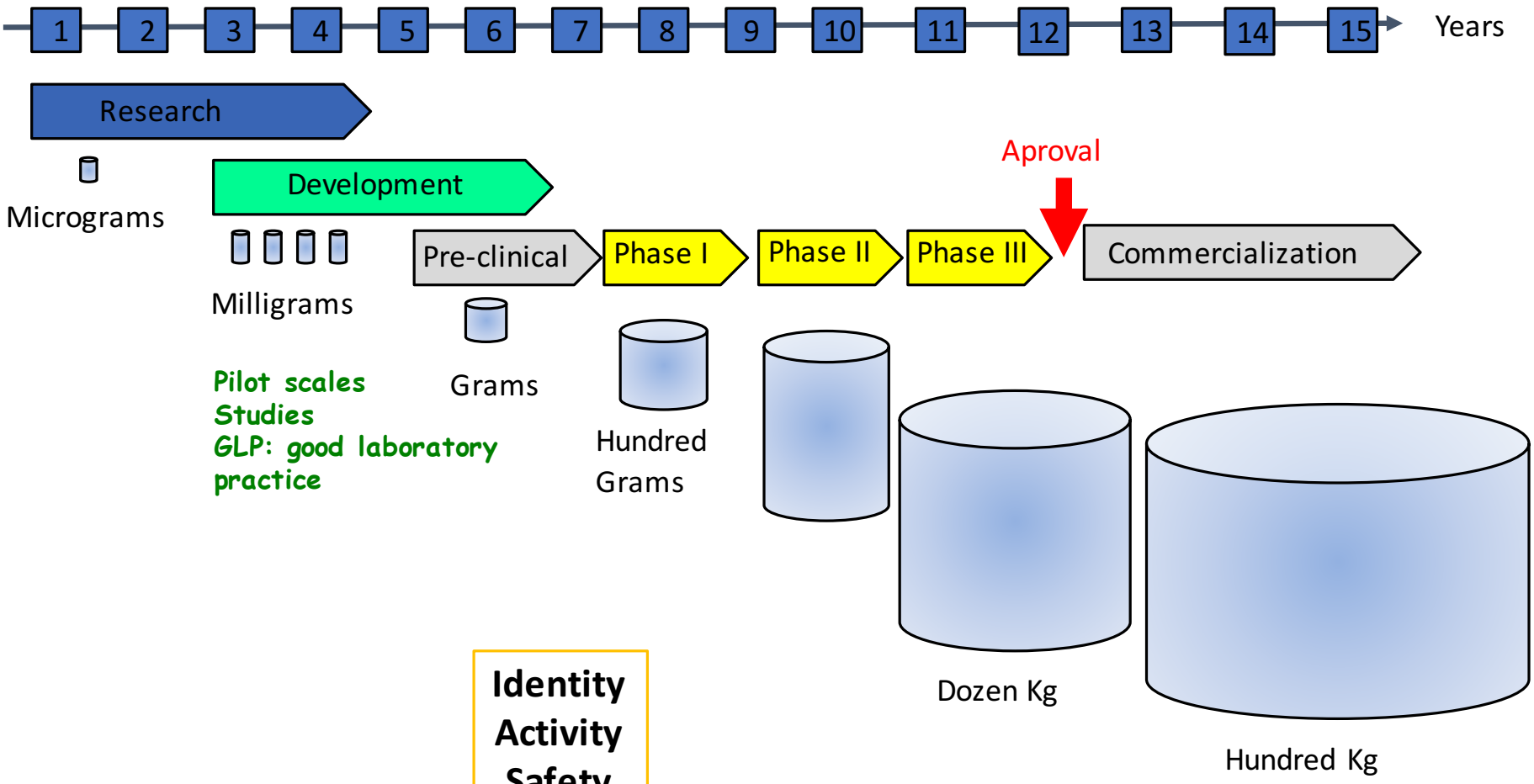
- ✓ Biological materials isolation
- ✓Modification
- ✓multiplication
  - ✓ Characterization & sequencing
  - ✓ Cloning
  - ✓ Expression

Genetic engineering

- ✓ Simple and robust process
- ✓Energy balance, raw materials, waste treatments
- ✓Regulatory environment

- ✓ Industrial production
- ✓ Purification and characterization
- ✓ Quality control
- ✓ Conditionning

From research to commercialization



General scheme of a production process

Upstream processing: biomass production

Initial culture
Expansion
Production

Extraction → Concentration/ Primary Purification → Purification Resolutive Chromatography

Downstream processing: Purification steps

Drug Substance

Formulation → Conditioning → Labelling Packaging Storage

Drug Product

Upstream processing (USP)

Productive strain choice

Rusticity: adaptability to industrial conditions

Performances: growth speed, production speed, production yield…

Genetic stability

Bioreactors, required functions:

- **Confining**
- **Sterility**
- **Mixing**
- **Aeration + air filtration**
- **Nutrient and fluid supply**
- **Cleaning procedures**

Microbial Culture parameters

Physical, chemical:
pH, pO2, T°, osmotic pressure

Kinetics:
Dilution, nutrients, culture length…

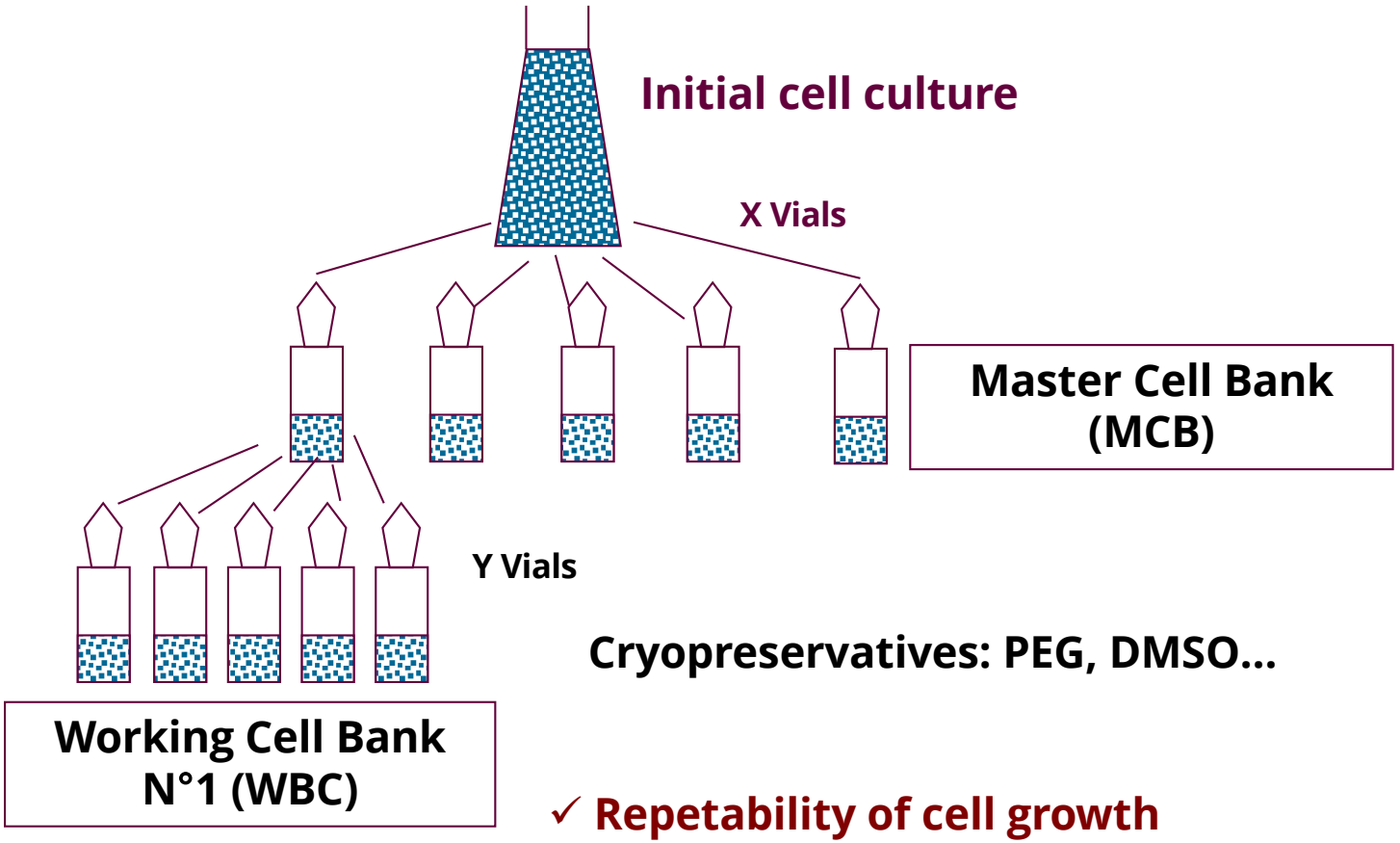
Nutrient composition :
Nitrogen, carbon, growth factors, lipids, oligoéléments (Mn, Fe, Co, Ni, Cu, Zn, Mo)…

Metabolit accumulation

Process modelisation and automation

BioBanking

Goal: to store and securize the well characterized inicial cell culture



Cryopreservatives: PEG, DMSO…

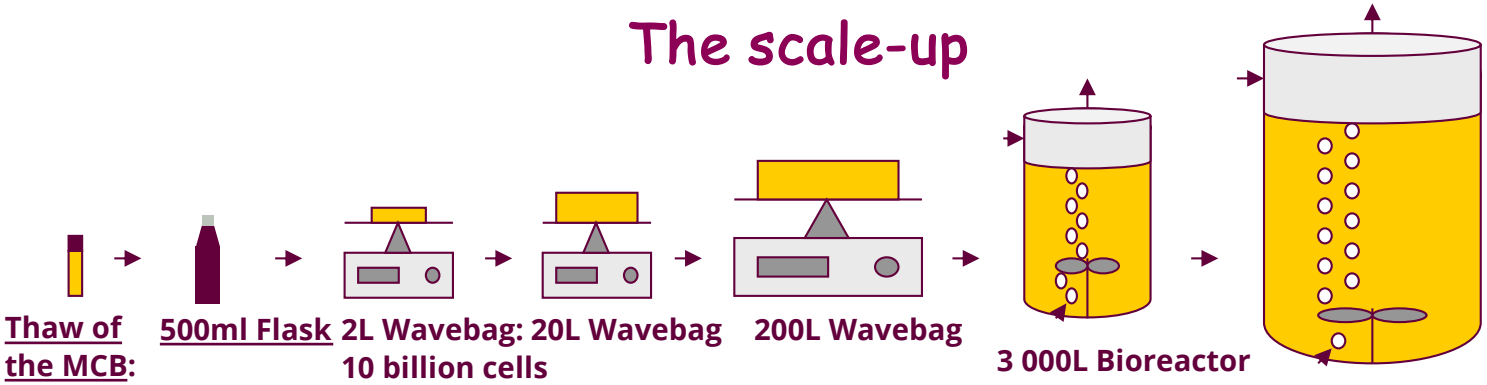
Quality control

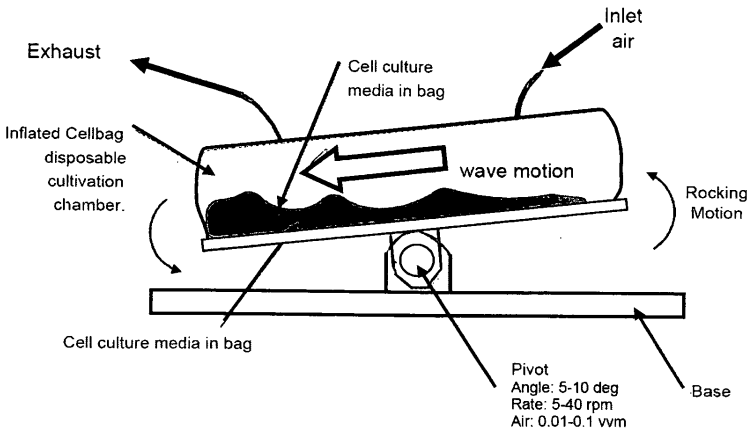
Cell bank characterization tests

Culture purity
Species identity
DNA sequencing
Plasmid identity and integrity
Host strain identity
Viable count
Specific productivity
Plasmid retention
Restriction endonuclease mapping

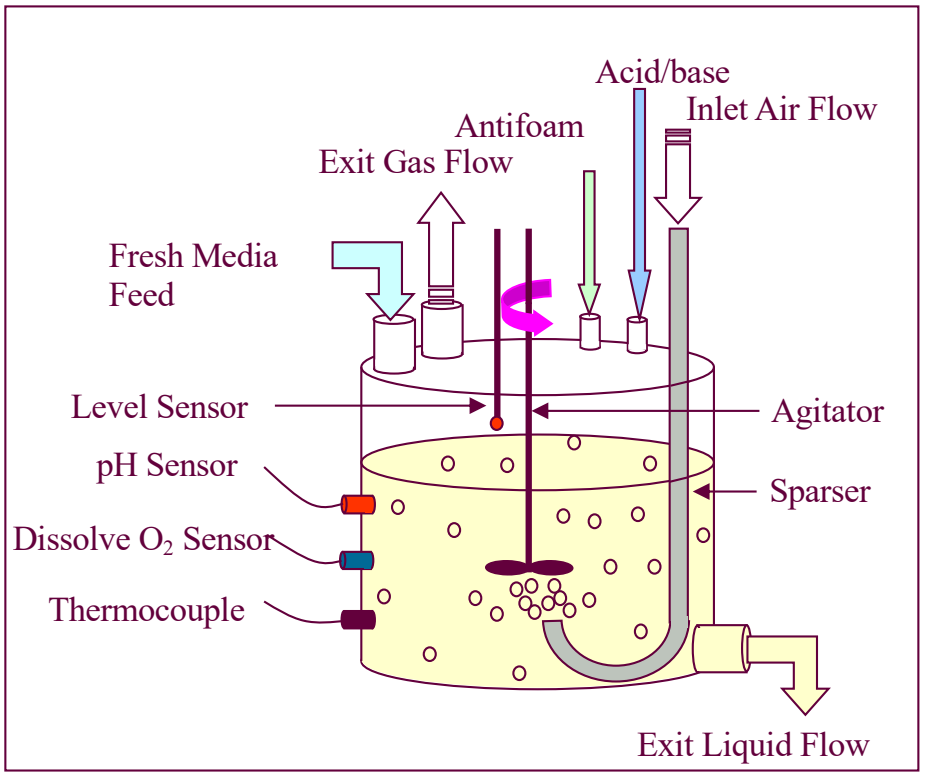
- ✓ Repetability of cell growth
- ✓ Quality control procedures and cell bank viability

The scale-up

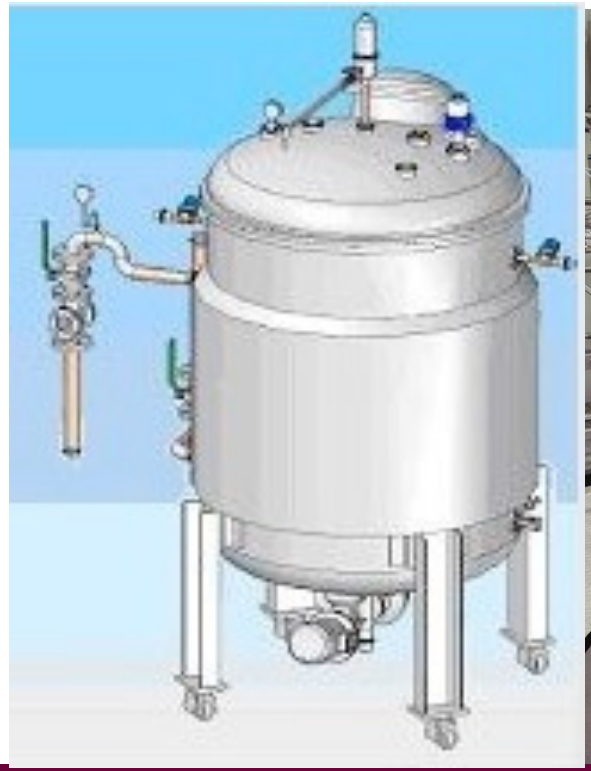




The« Wave » technology



Stirred-tank bioreactor



3 production modes

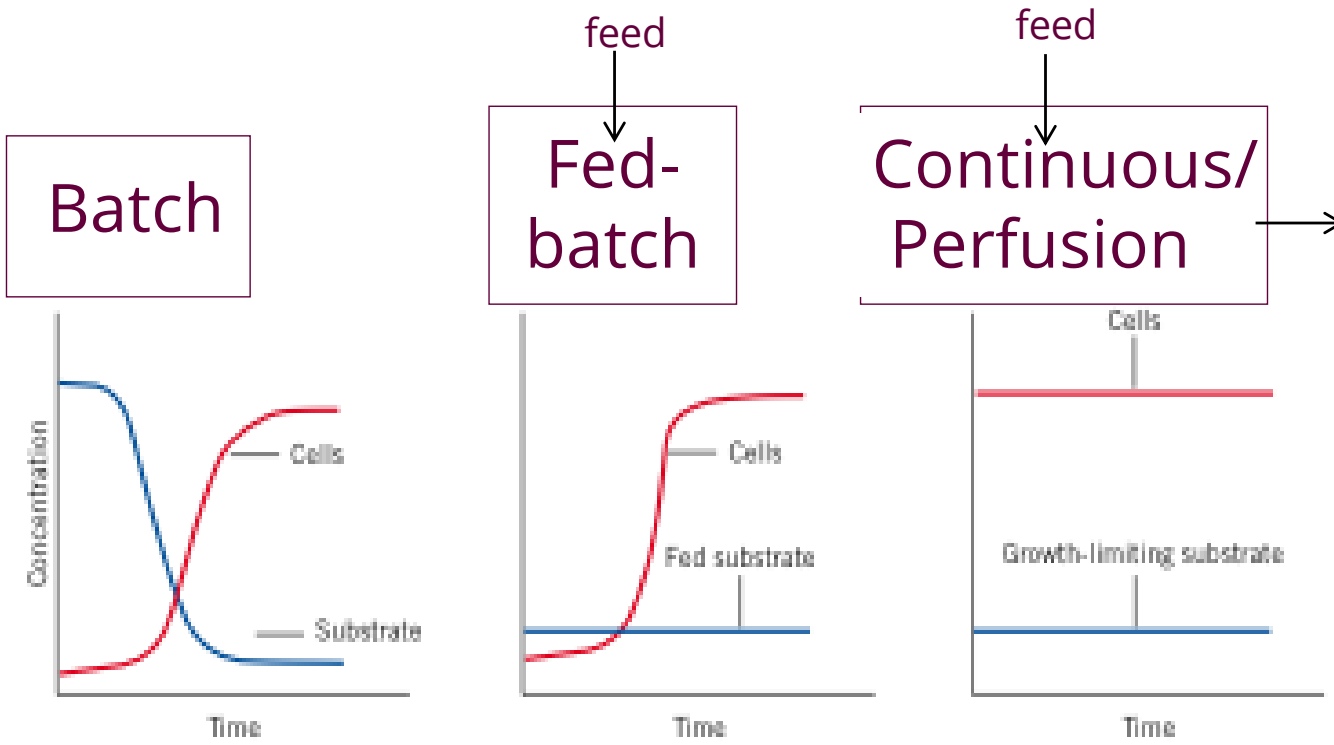


Fig. 95. Comparison of batch and perfusion concerning nutrients and waste products.

Continuous: removal of cells at the same rate

Perfusion: cells are retained or recycled

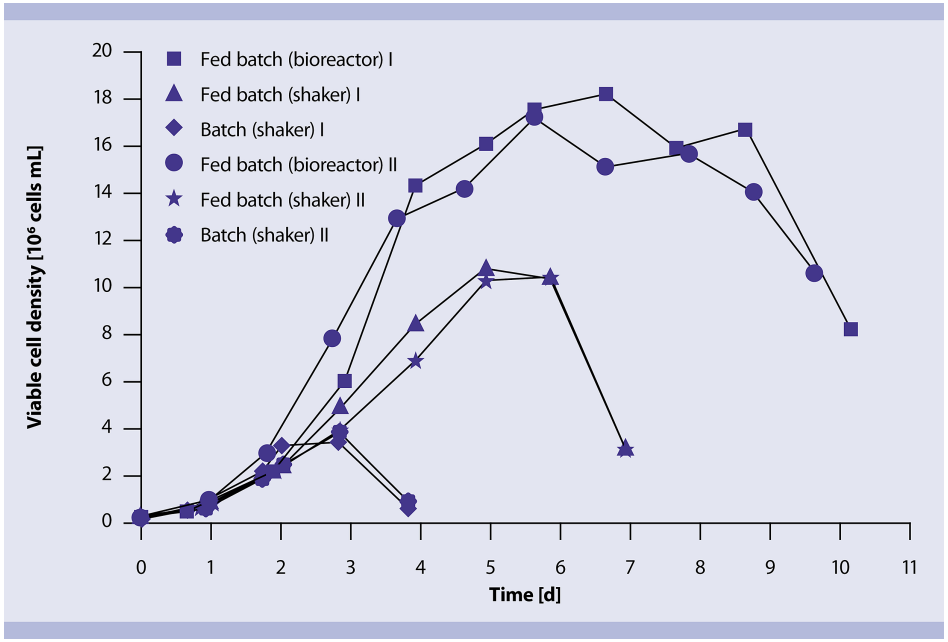
✓Variable medium composition
✓Toxic metabolite accumulation

✓Increase cell density
✓Easy automation
✓Bioproduct accumulation

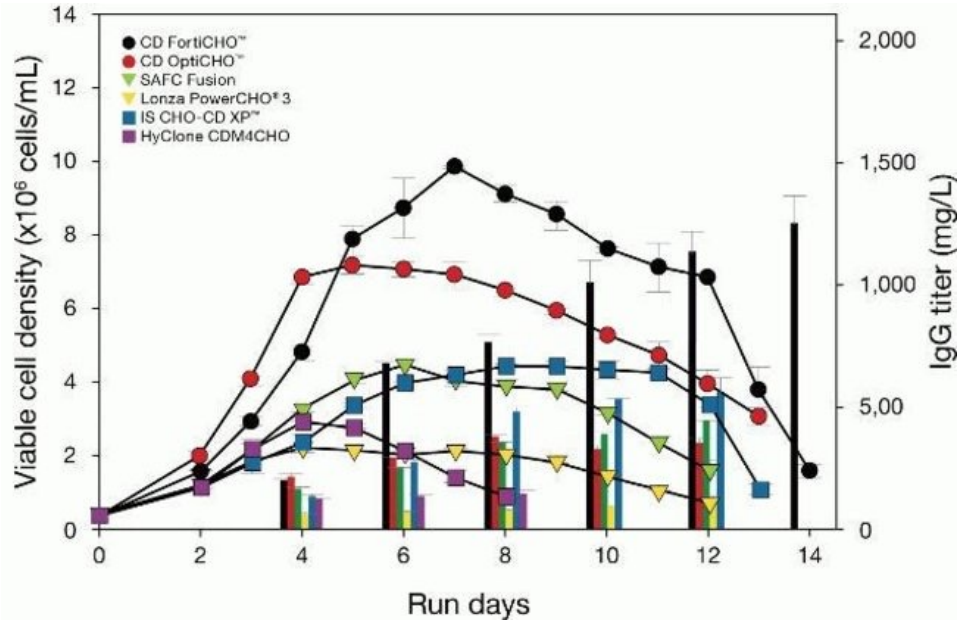
✓Metabolite elimination
✓Stable medium composition
✓Reduced degradation risk

Process development USP – cell growth optimization

Bioreactor comparison



Cell growth media comparison



Critical issues by regulatory authorities:

- ✓ **safety,**
- ✓ **genetic and phenotypic stability,**
- ✓ **monoclonality of the production cell line**

➔ Rigorous cell line development:
Full documentation
Full characterization of the final production cell line

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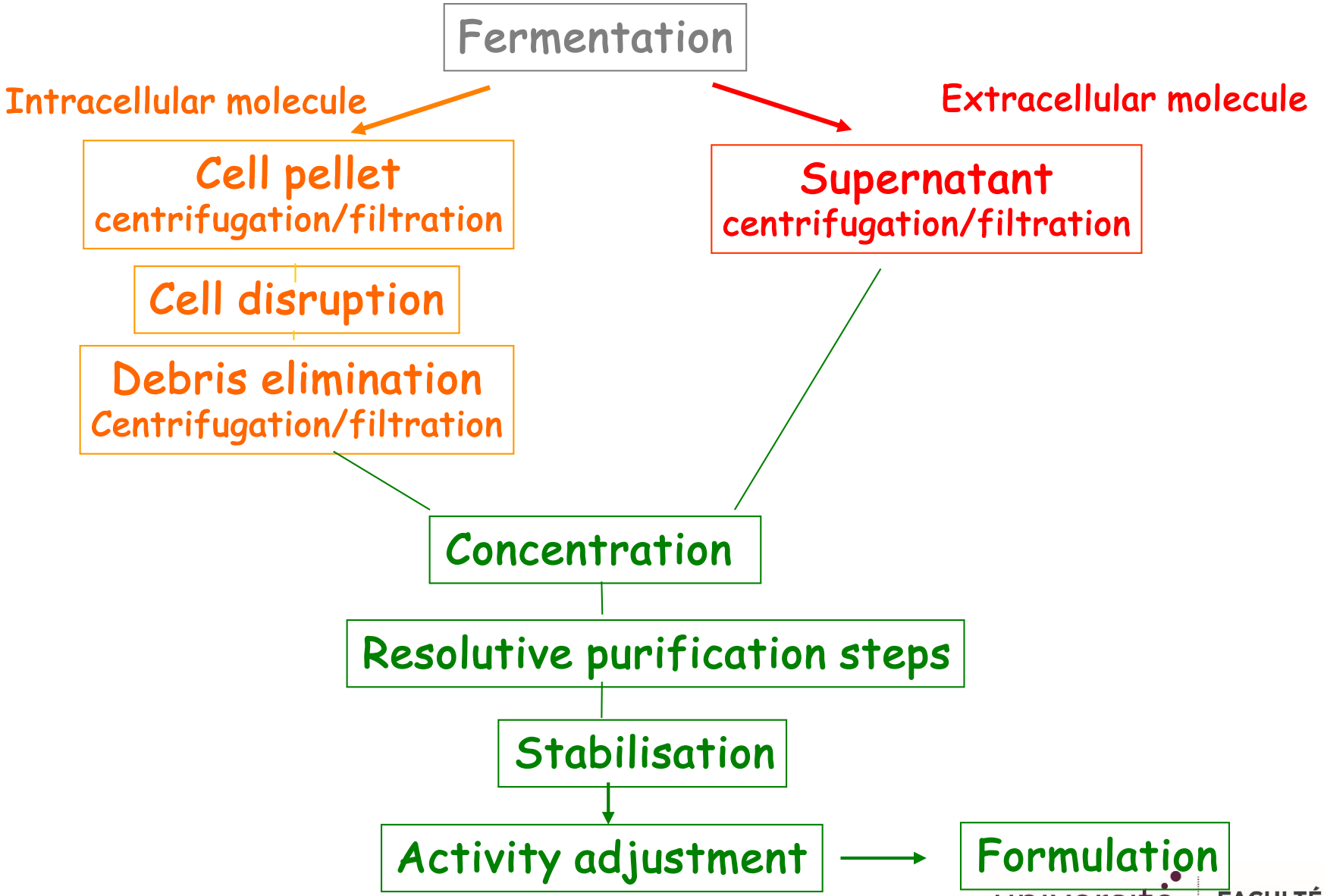
Downstream processing (DSP)



Centrifuge

Filtration installation

Chromatography

Example 1:

Antibiotic production

Main AB producers

Secondary metabolites: stationnary phase synthesis

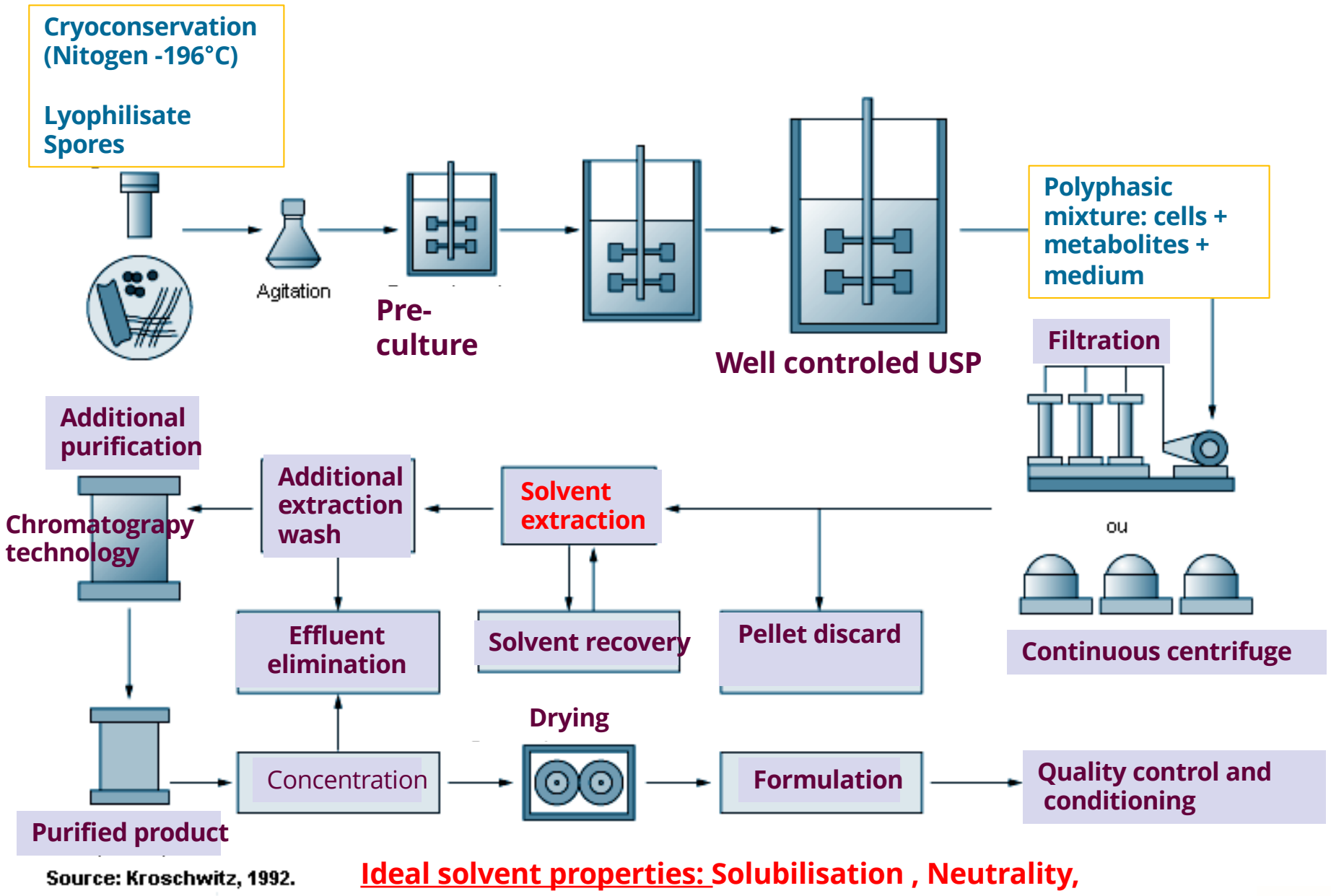
80% Bacteria, steptomyces

Bacillus

Fungi
Aspergillus, Penicillium, Cephalosporium, Heminthosporium, Fusidium

Classification	Antibiotique	Organisme producteur
Aminoglycosides	Streptomycine	*S. griseus*
	Spectinomycine	*S. spectabilis*
	Néomycine B	*S. fradiae*
Tétracyclines	Chlortétracycline	*S. aureofaciens*
	Oxytétracycline	*S. rimosus*
Polyènes	Nystatine	*S. noursei*
	Amphotéricine	*S. nodosus*
Macrolides	Spiramycine	*S. ambofaciens*
	Érythromycine	*S. erythreus*
	Rapamycine	*S. hygroscopicus*
	Natamycine	*S. natalensis*
	Avermectine	*S. avermitilis*
	Tylosine	*S. fradiae*
	Oléandomycine	*S. hygroscopicus*
Streptogramines	Pristinamycine	*S. pristinaespiralis*
	Virginiamycine	*S. virginiae*
Glycopeptides	Vancomycine	*S. orientalis*
Lincosamides	Lincomycine	*S. lincolnensis*
Autres	Chloramphénicol	*S. venezuela*

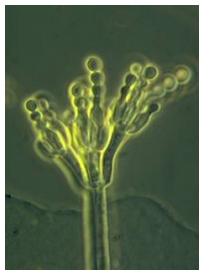
General Scheme for AB production

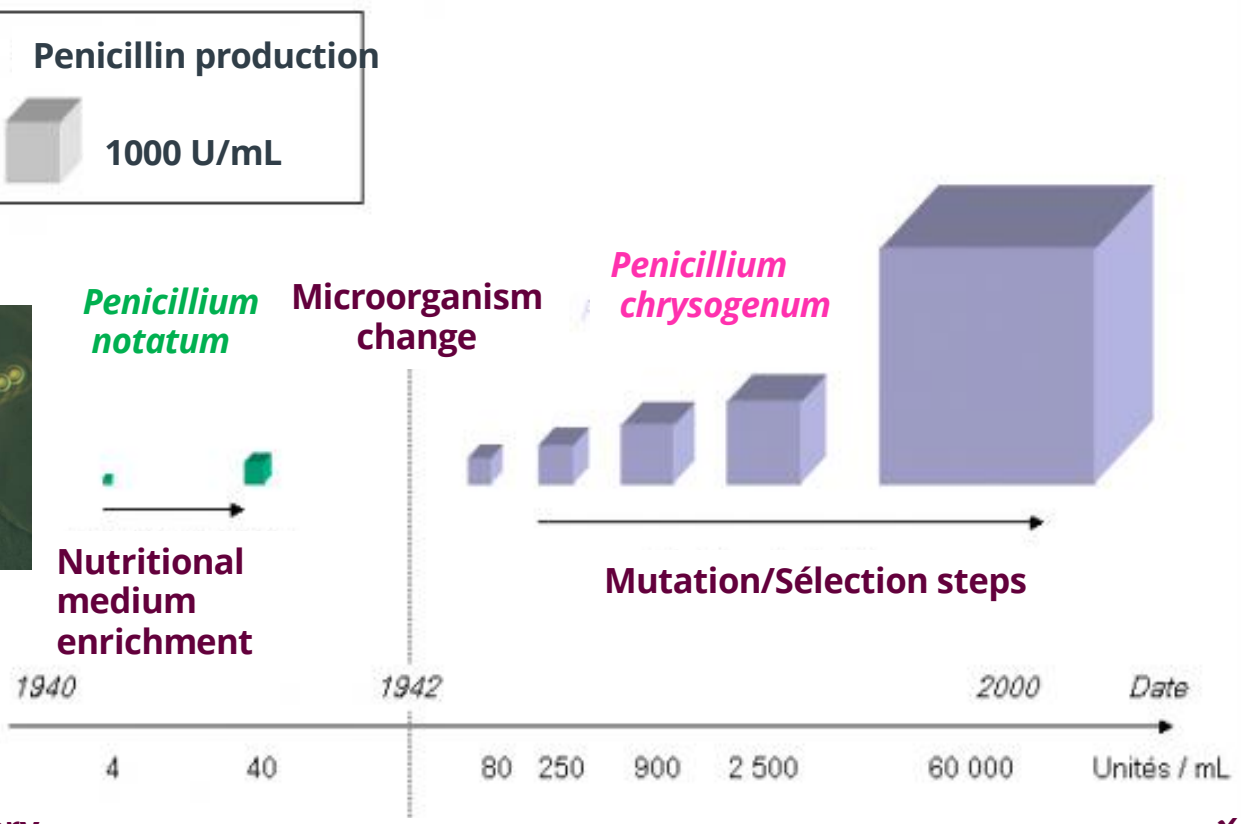


Source: Kroschwitz, 1992.

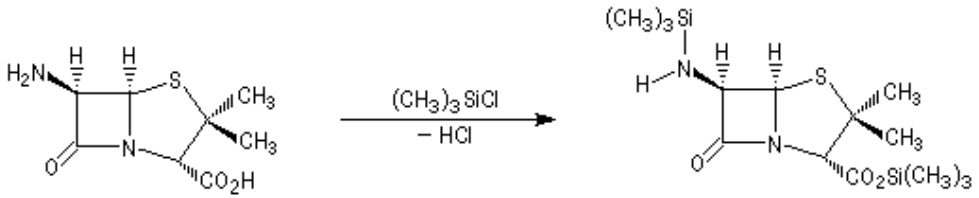
Ideal solvent properties: Solubilisation , Neutrality, Selectivity, Moderate cost, Elimination

- **Penicillin example**





Discovery
1897 (E Duchesne)
1928 (A. Fleming)

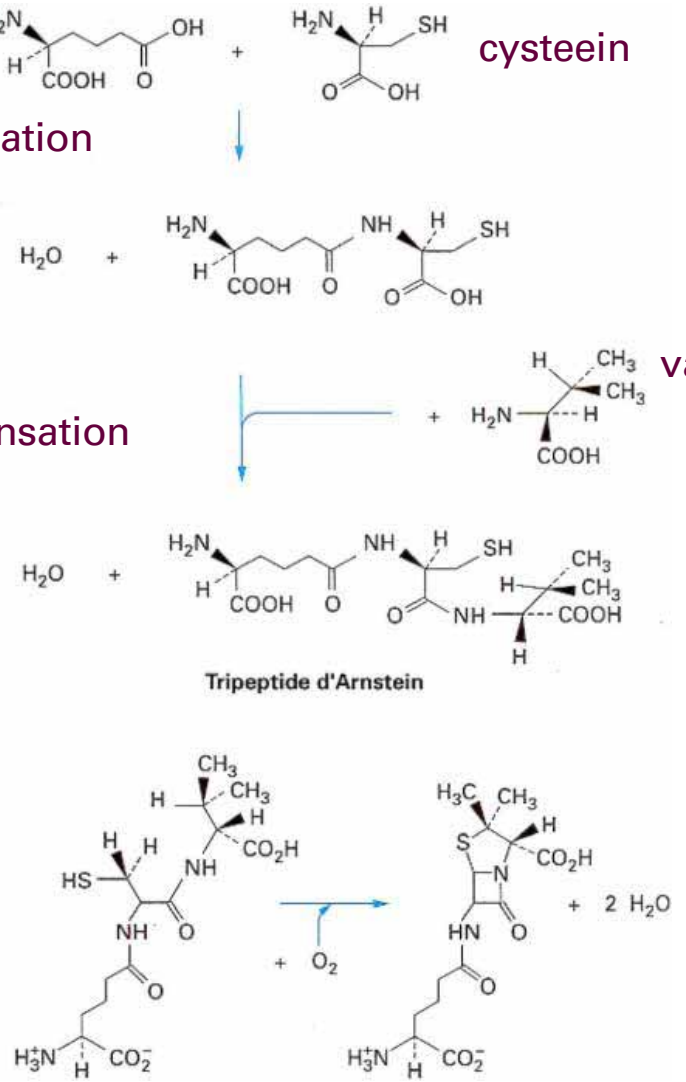


- **Production pH≈7**
- **Penicillin excretion at early stationnary phase;**
- **Organic solvent extraction**
- ✓ **Aqueous phase**
- ✓ **Penicillin cristallisation**
- ✓ **Hémisynthesis: transformation into derivates AB: ampicillin amoxicillin…**

Penicillin biosynthesis secondary metablism ➔ end of cell growth

aminoadipic acid + cysteein

condensation



Oxidative cyclisation ➔ isopenicillin N

Quality control of antibiotics

Final step = dessication (=lyophilisation)

Essays described in the Europena Pharmacopeia

- **Identification**
- **Impurity dosage: derived molecules, pyrogenic substances, histamin…**
- **Activity**

EUROPEAN PHARMACOPOEIA ONLINE

edqm
European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

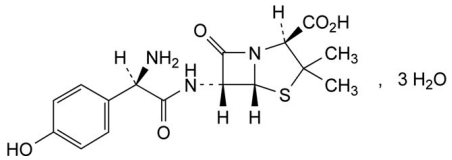
Example with amoxicillin

EUROPEAN PHARMACOPOEIA ONLINE

Available With Paris-Saclay Library

AMOXICILLIN TRIHYDRATE

Amoxicillinum trihydricum



$C_{16}H_{19}N_3O_5S,3H_2O$ M_r 419.4
[61336-70-7]

DEFINITION

(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-Amino-2-(4-hydroxyphenyl)acetyl]-amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate.

Semi-synthetic product derived from a fermentation product.

Content: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

Appearance: white or almost white, crystalline powder.

Solubility: slightly soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in fatty oils. It dissolves in dilute acids and dilute solutions of alkali hydroxides.

IDENTIFICATION

First identification: A.

Second identification: B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).

Comparison: *amoxicillin trihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

Test solution. Dissolve 25 mg of the substance to be examined in 10 mL of *sodium hydrogen carbonate solution R*.

Reference solution (a). Dissolve 25 mg of *amoxicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

Reference solution (b). Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

Plate: *TLC silanised silica gel plate R*.

Mobile phase: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

Application: 1 µL.

Development: over a path of 15 cm.

Drying: in air.

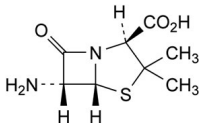
Detection: expose to iodine vapour until the spots appear and examine in daylight.

System suitability: reference solution (b):

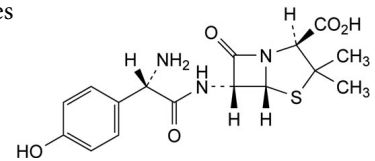
– the chromatogram shows 2 clearly separated spots.

Results: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

IMPURITIES

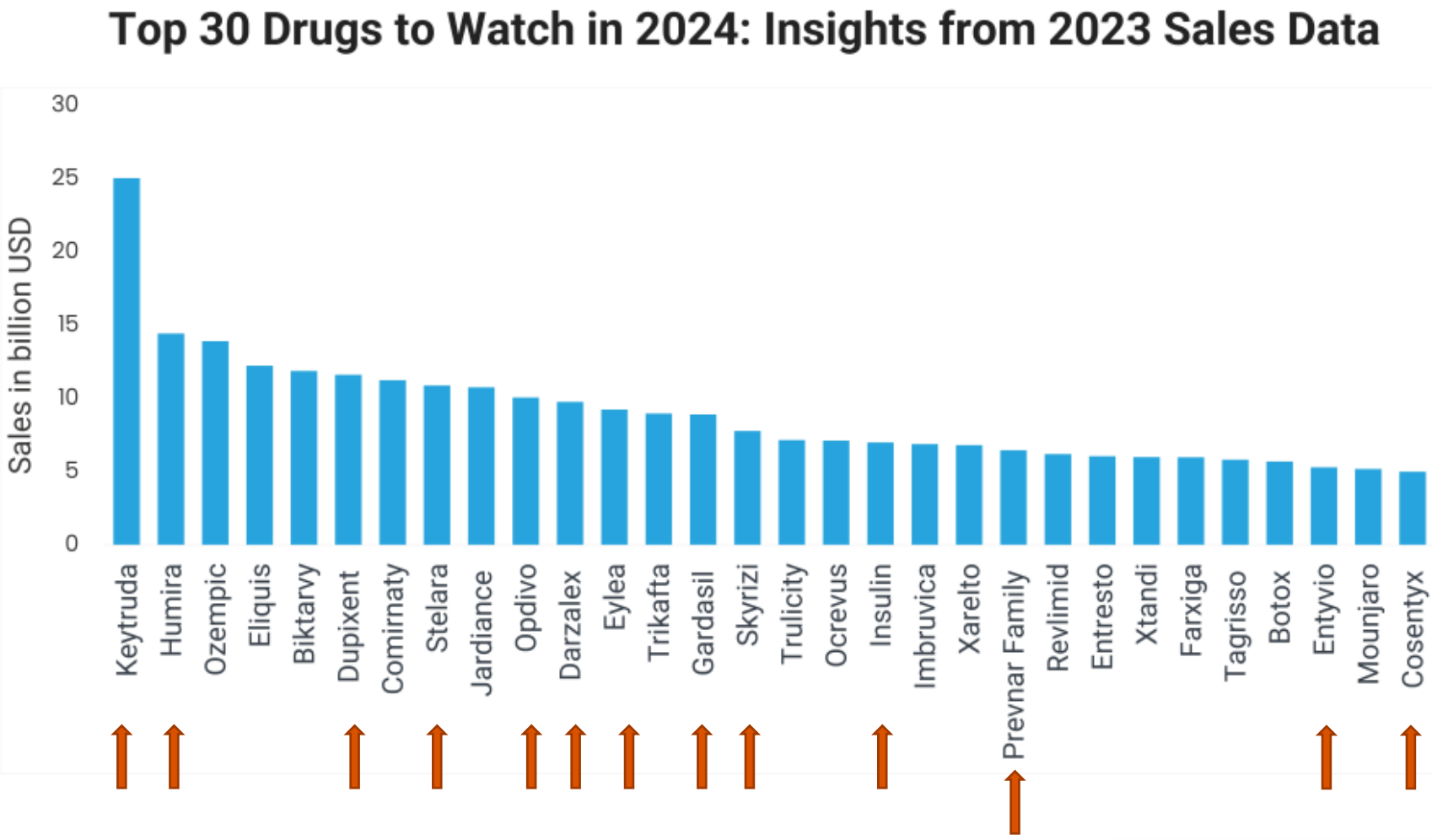


A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),



B. (2*S*,5*R*,6*R*)-6-[[(2*S*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid (L-amoxicillin),

Example 2:
Recombinant protein production



https://xtalks.com/top-30-drugs-to-watch-in-2024-insights-from-2023-sales-data-3908/

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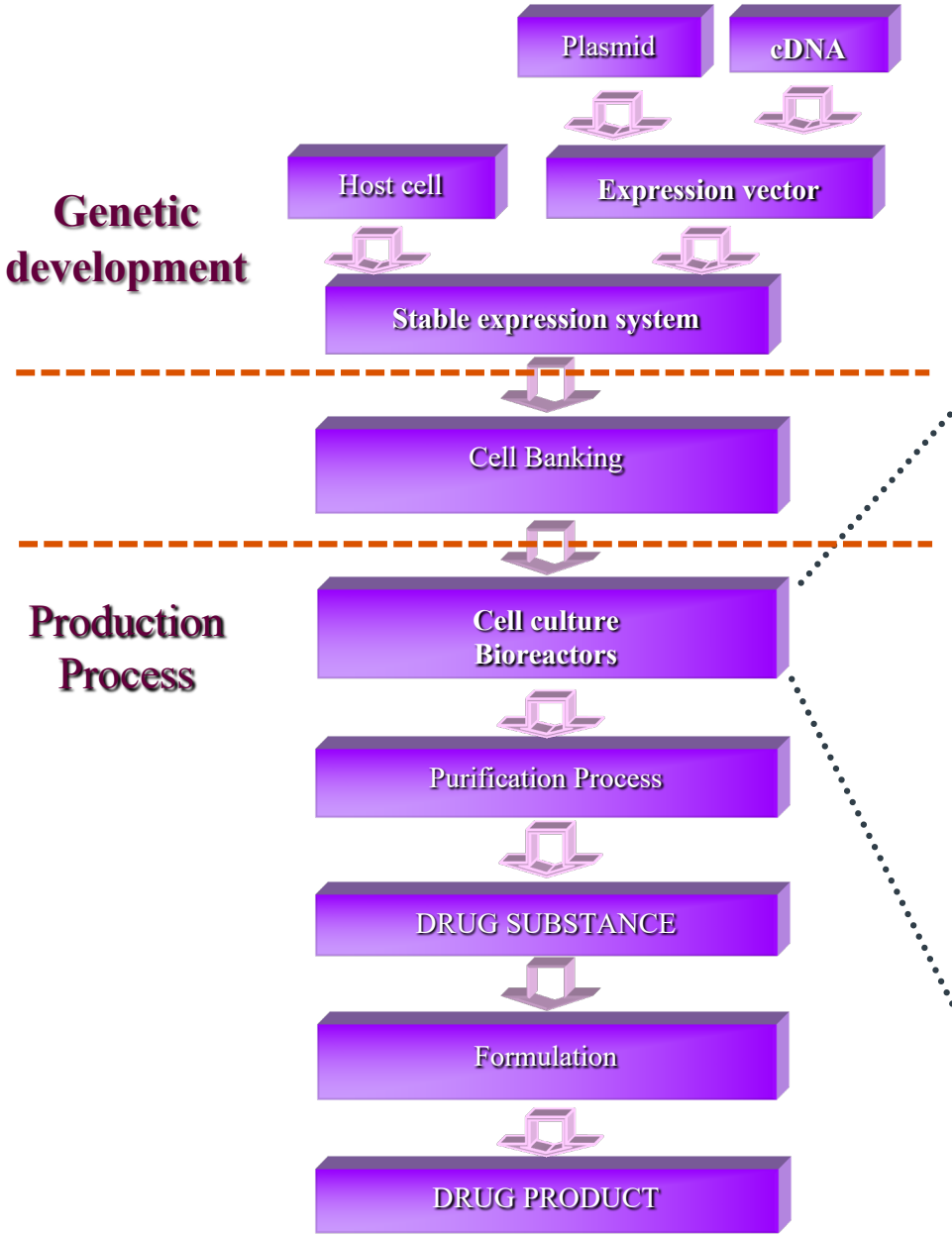
Recombinant Protein production, an overview

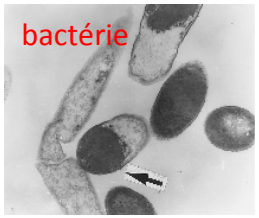


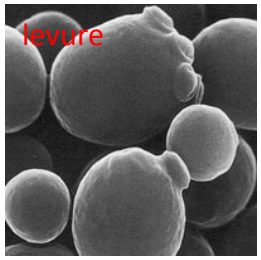
200mL roll bottle

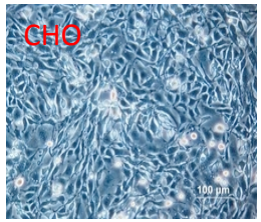
20 L wavebag

10 000 L Bioreactor

Recombinant Protein technology, the cell line development







cDNA sequence

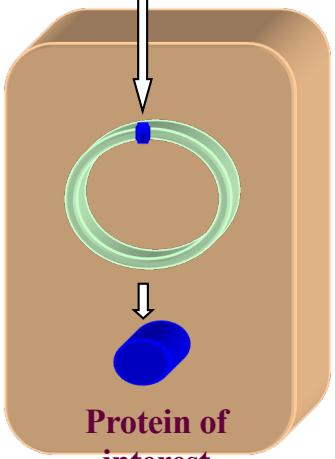
plasmid

Insertion

Expression vector

Transfection

Genetically modified organism = Host cell



Objective:
to generate THE producing cell

- ✓ Expression vector optimization
- ✓ Targetting the insersion site

- ✓ Transfected cells selection
- ✓ High producers selection
- ✓ Cell line genetic caracterization
- ✓ Culture condition optimization

- **GMO choice**
- **Protein expression level**
- **Protein properties**

Industrial production (cell factory)

	E. Coli	Yeast	Mammalian	Insect
Proteolytic clivage	**?**	**?**	**yes**	**yes**
Glycosylation	**non**	**?**	**yes**	**?**
Secretion	**?**	**yes**	**yes**	**yes**
Folding	**?**	**?**	**yes**	**yes**
Phosphorylation	**No**	**?**	**yes**	**?**
Acetylation	**No**	**yes**	**yes**	**?**
Amidation	**No**	**yes**	**yes**	**yes**
% P / total	**>50%**	**1%**	**<1%**	**>30%**
MM, quantity	**60-70 kDa 100g/L**	**30kDa 10g/L**	**<300kDa 1-5g/L**	**60kDa 200g/L**

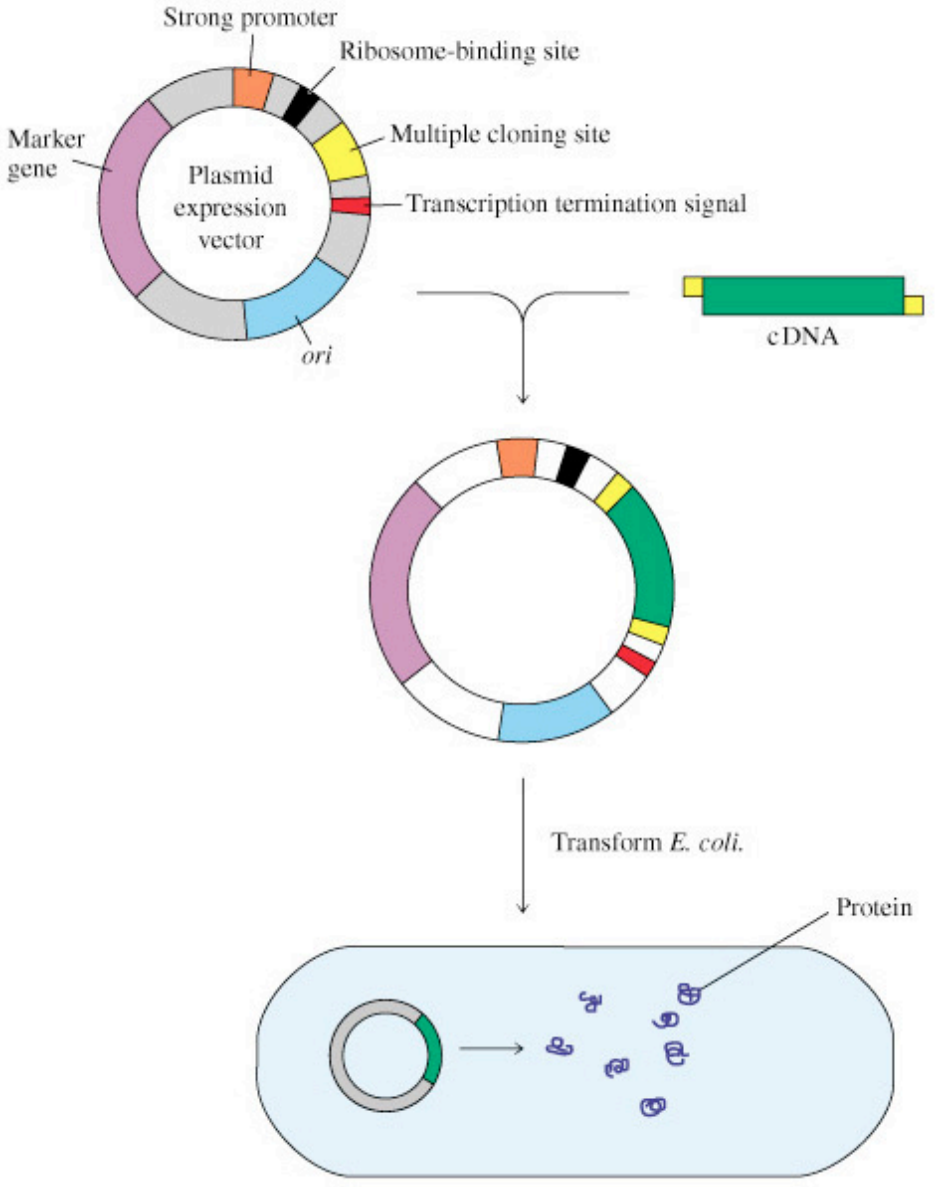
Protein	System	Production level
Hirudin	*S. cerevisiae* (Y)	60 mg/L
	H. polymorpha (Y)	–
Interferon α-2b	*H. polymorpha* (Y)	120 mg/L
Hepatitis B vaccine	*H. polymorpha* (Y)	–
Angiostatin	*P. pastoris* (Y)	108 mg/L
Anti-HBs Fab	*P. pastoris* (Y)	50 mg/L
Human serum albumin	*K. lactis* (Y)	3 g/L
	S. cerevisiae (Y)	3 g/L
	P. pastoris (Y)	10 g/L
Human interleukin 6	*A. niger* (F)	150 mg/L
Human apolipoprotein AI	CHO cells (M)	80 mg/mL
Insulin precursor	*P. pastoris* (Y)	3 g/L
	S. cerevisiae (Y)	98 mg/L
Human tPA	CHO cells (M)	34 mg/L
Human gonadotropin	CHO cells (M)	3 g/L
Erythropoietin (epoetin α)	CHO cells (M)	–
Monoclonal Ab	NSO cells (M)	3 g/L
HPV vaccine (Cervarix™)	Insect cells	–
Human proapolipoprotein AI	Insect cells	80 mg/L
Clotting factor VIIa	BHK cells (M)	–

Current Opinion in Biotechnology 2012, 23:965–971

Protein production in Bacteria (*E.coli)*

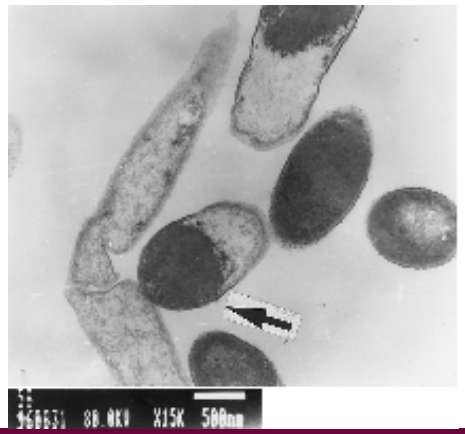
Host bacteria characteristics:

- recA (-)
- dam ou dcm (-)
- No endogenous plasmid



Intra cellular production

- ✓ Few folding proteins
- ✓ No post-tranpost-translational modification
- ✓ Reducing environment



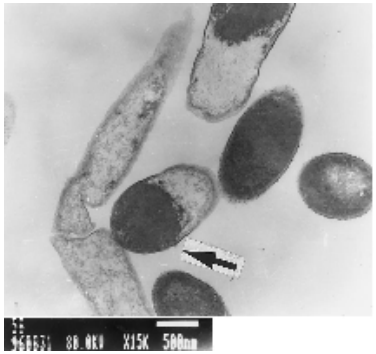
Inclusion bodies =protein precipitation

PROS

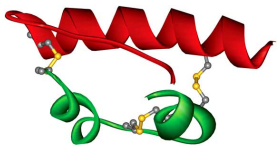
- Fast and inexpensive growth
- Well-known genetic system, many improved strains
- Controllable gene expression
- Many expression vectors available, ease of transfection into host bacteria
- Good protein production yields (>10 g/L culture)
- Cytoplasmic inclusion bodies: purification ease

CONS

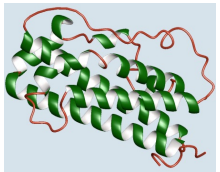
- No post-translational modification
- Inclusion body: insoluble, poorly folded protein
- Presence of bacterial endotoxins: the purification process must include elimination and control steps



A few therapeutic proteins produced in *E Coli*:



Insulin
16kDa



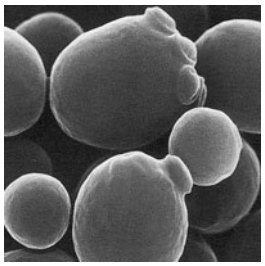
Growth hormone
22kDa

Interferons
20 kDa

ntibody fragment
5 kDa

Fab

Protein production in Yeast



Saccaromyces cerevisiae

Pichia pastoris

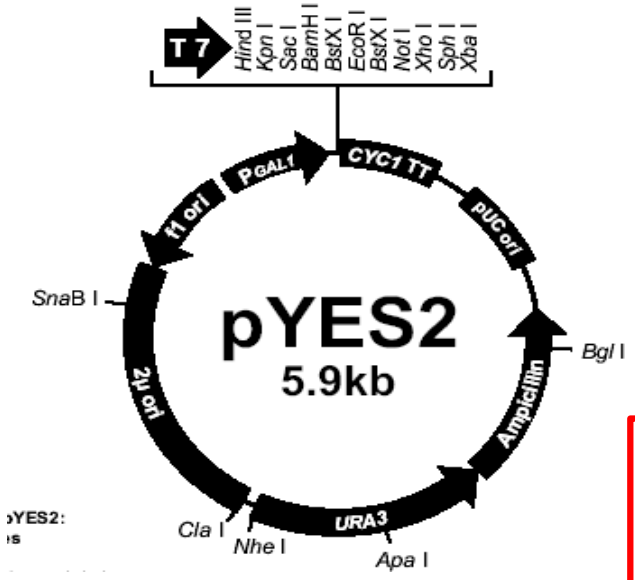
PROS

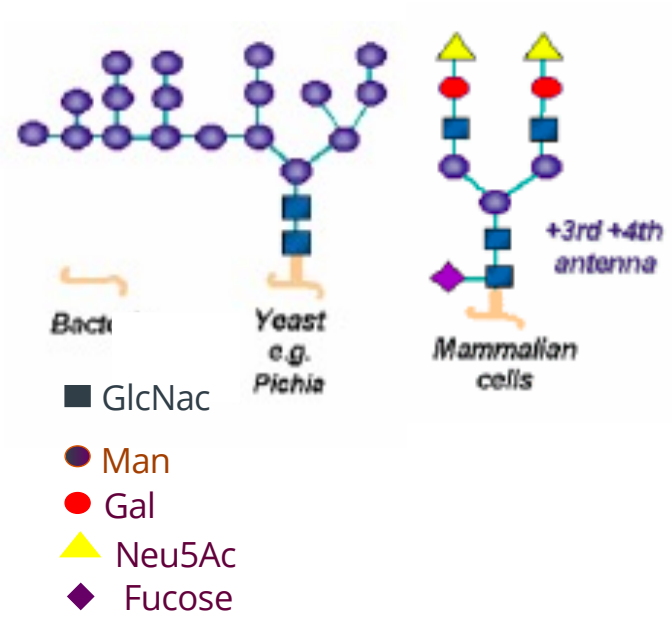
- ➢Small, eukaryotic genome and well characterized
- ➢ Absence of endotoxin
- ➢ Inexpensive fermentation
- ➢ Good yields (grams per liter of culture)
- ➢ Simple post-translational modifications
- ➢Secretion of the protein of interest

INCONVENIENTS

- ➢**Hypermannosylations**
- ➢**Folding**

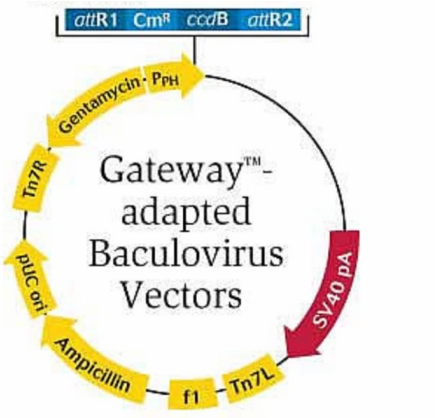
Glyco-engineerinf for humanization





Proteins produced in yeast
Insulin
GM-CSF
Vaccine HBV
Vaccine HPV
Glucagon

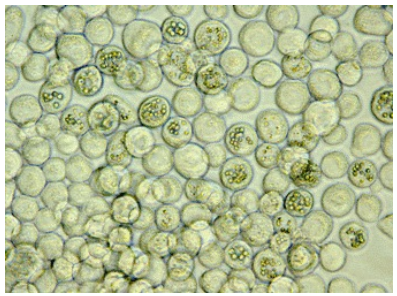
Protein production in insect cells



Baculovirus: insect cell virus

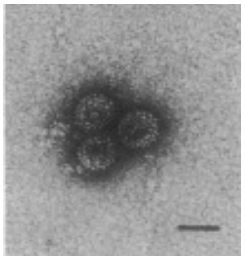
➔ Use of recombinant baculovirus (bearing the cDNA sequence of interest) to infect insect cell suspension

➔ Protein synthesis



➢**CERVARIX® (EMA, 2007 and FDA, 2009) bivalent vaccine bivalent against papillomavirus: prévention of cervical cancer**

HPV-16 L1 protein + HPV-18 L1 protein



Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic

R. KIRNBAUER*, F. BOOY†, N. CHENG†, D. R. LOWY*, AND J. T. SCHILLER*

Proc. Natl. Acad. Sci. USA, 1992

➢**PROVENGE® (FDA, 2010)**

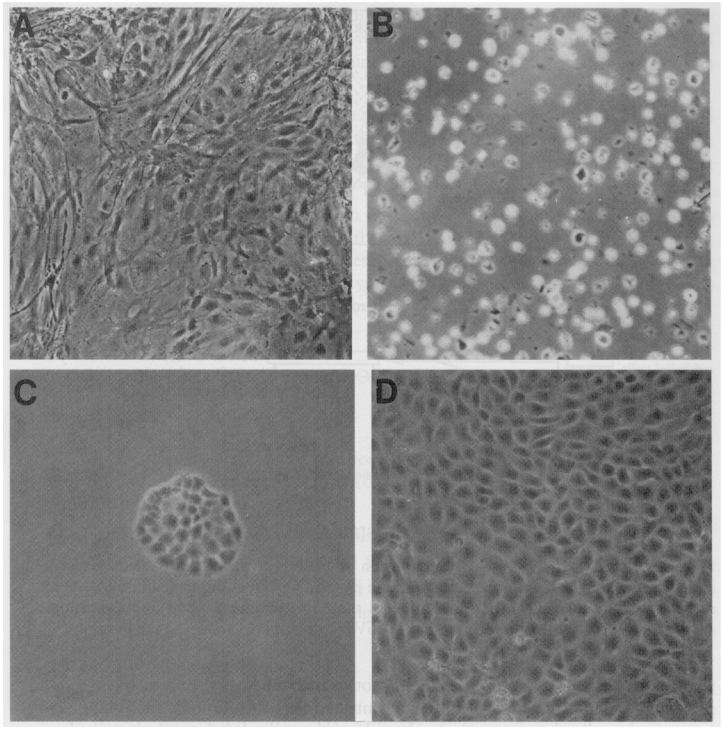
Autologous product for prostate cancer cell therapy: PSA produced in *S. frugiperda*

➢**FluBlok® (FDA, 2011)**

Recombinant influenza vaccine➔ Time and cost-reduced compared to egg production protocol

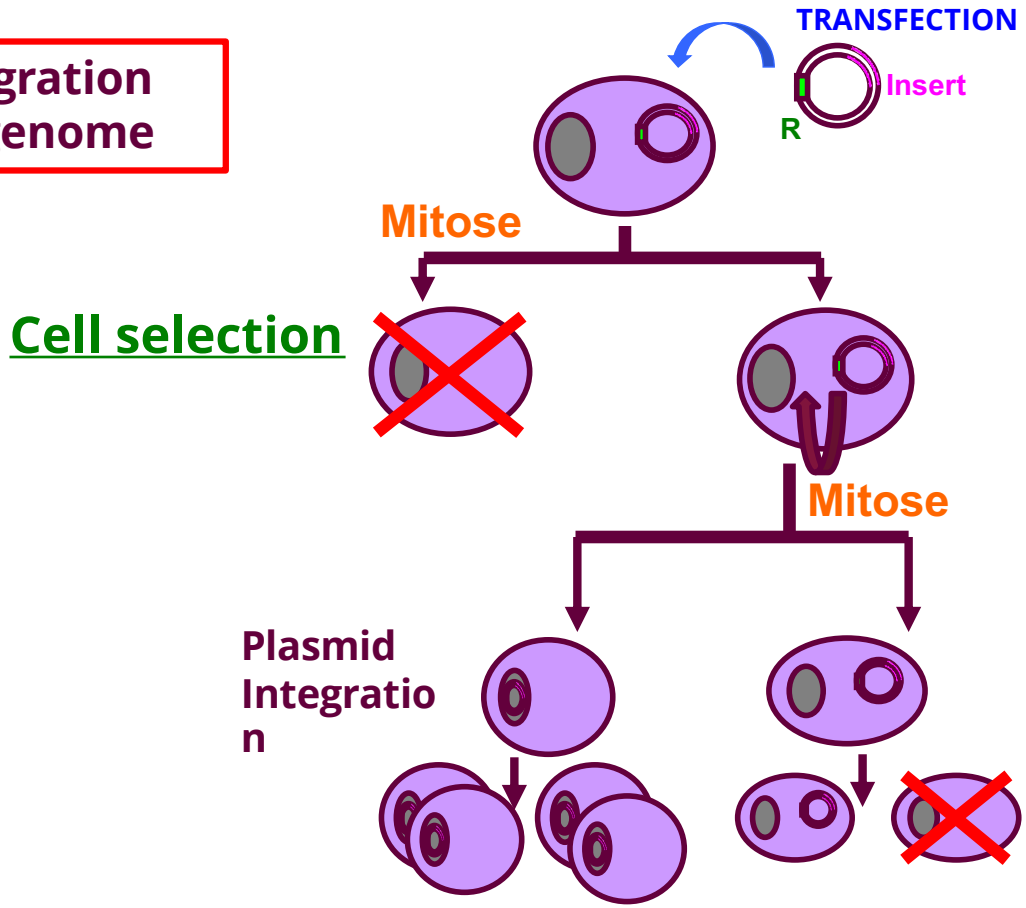
Protein production in mammalian cells

Vector integration in the cell genome



A Non transfected cells

B-D Transfected cells with selection pressure



No integration: plasmid loss

➔ Stable cell line for protein production

Secretion of the protein

Tight quality control of the cell-line

Protein production in mammalian cells

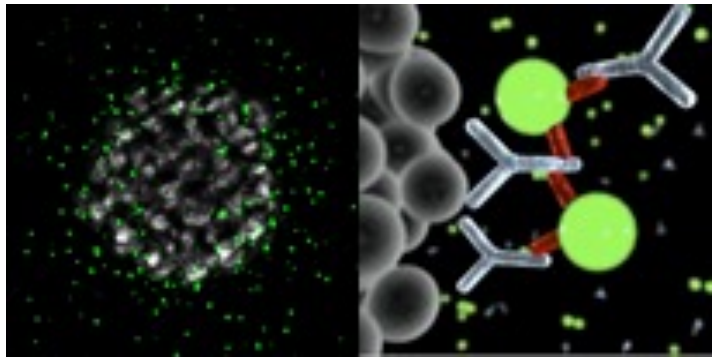
ECACC : European Colection of Cell Culture www.ecacc.org.uk

Cell	Biological source	Some common uses
CHO	Chinese Hamster Ovary cells	Recombinant proteins
VERO	Kidney epithelial cells extracted from an African green monkey	Human and veterinary viral vaccines (rotavirus, rabies, poliovirus)
BHK	Baby Hamster Kidney fibroblasts	Factor VIII, veterinary viral vaccines
HEK 293	Human embryonic kidney	Adenoviral vectors (SARS-CoV2)
Hybridomas	Murine hybrid cell line	Monoclonal antibodies
PER.C6	Derived from human embryonic retinal cells	Recombinant proteins, adenoviral vectors (SARS-CoV2)
NS0	Derived from the non-secreting murine myeloma	Recombinant proteins
MDCK	Madin-Darby canine kidney cells	Viral vaccines (flu)
MRC5	Human fetal lung fibroblast cells	Human viral vaccines (flu)

https://www.mabdesign.fr/wp-content/uploads/2021/07/Immunowatch-Edition-3-Bioproduction-2.pdf

Best producing clones selection

http://www.genetix.com



CHO → Transfection → selection → **Clonality**

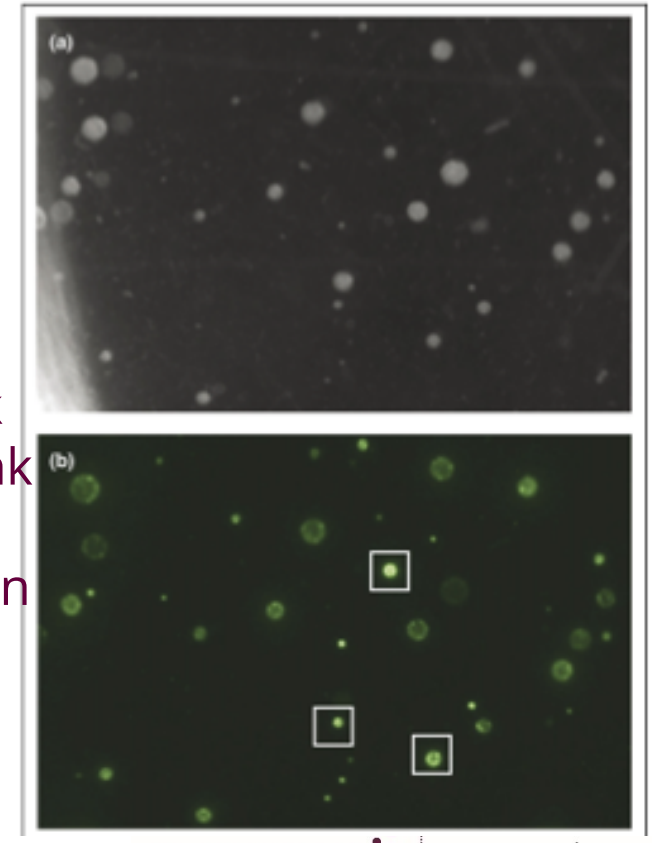
Clonality → Cell growth *Serum-free medium* → Clones selection

Clones selection → Clone characterization :

- ✓ Transgene copy number (qPCR)
- ✓ Cell identity
- ✓ Cell viability
- ✓ Cell purity…

Clone characterization :

- ✓ Master Cell Bank
- ✓ Working Cell Bank
- ✓ End of Production



Browne et al. Trends in Biotechnology. 2007

Quality control of the producing cell cultures, an example

The first recombinant human coagulation factor VIII of human origin: human cell line and manufacturing characteristics

Casademunt et al., European Journal of Haematology. 2012; 89 (165–176)

Table 2 Overview of tests performed and results obtained

	MCB	WCB	End of production cells	Bulk harvest
Identity				
Isoenzyme analysis	HEK 293	–	–	–
RAPD	HEK 293	HEK 293	HEK 293	–
Microbiological tests				
Sterility	Complies	Complies	Complies	Complies
Mycoplasma	Negative	Negative	Negative	Negative
Viral tests				
In vitro assay for the detection of adventitious viruses (28 d, three detector cell lines: Vero, MRC5 and 293)	Negative	–	Negative	Negative
In vivo assay for the detection of adventitious viruses (adult and suckling mice, embryonated eggs)	Negative	–	Negative	Negative
QF-PERT	Negative	–	Negative	Negative
PCR screen for human viruses (HIV 1/2, HTLV 1/2, CMV, EBV, HHV 6/7/8, HBV, HCV, B19, HPV, HPyV)	Negative	–	–	–
PCR screen for AAV-2	Negative	Negative	Negative	Negative
TEM	No virus detected	–	No virus detected	No virus detected
MMV infectivity assay	Negative	–	–	–
In vitro bovine virus screen (BVDV, BAV, BRSV, BPV, REO3, BTV, RV)	Negative	–	–	–
PCR screen for bovine polyoma virus	Negative	–	–	–
In vitro porcine virus screen (PPV, PAV, TGE, HEV)	Negative	–	–	–

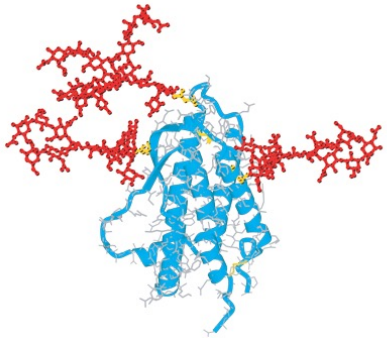
Cell identity

Microbiological tests

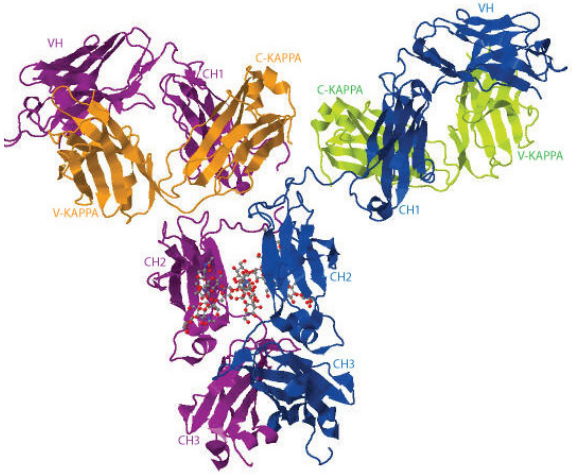
Viral security

MCB, master cell bank; WCB, working cell bank; EPC, end of production cells; BH, bulk harvest; RAPD, random amplified polymorphic DNA; QF-PERT, quantitative fluorescent product-enhanced reverse transcriptase; PCR, polymerase chain reaction; HEK, human embryonic kidney; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; CMV, cytomegalovirus; EBV, Epstein–Barr virus, HHV, human herpes virus; HBV, hepatitis B virus; HCV, hepatitis C virus; B19, human parvovirus B19; HPV, human papilloma virus; HPyV, human polyoma viruses JC and BK; AAV, adeno-associated virus; TEM, transmission electron microscopy; MMV, mouse minute virus; BVDV, bovine viral diarrhoea virus; BAV, bovine adenovirus; BRSV, bovine respiratory syncytial virus; BPV, bovine parvovirus; REO, reovirus; BTV, bluetongue virus; RV, rabies virus; PPV, porcine parvovirus; PAV, porcine adenovirus; TGE, transmissible gastroenteritis virus; HEV, haemagglutinating encephalitis virus.
– indicates tests have not been performed.

A few proteins produced in mammalian cell lines



Erythropoïétin (EPO)
165 aa, MM : 30,6 kDa ; 40% glycosylation

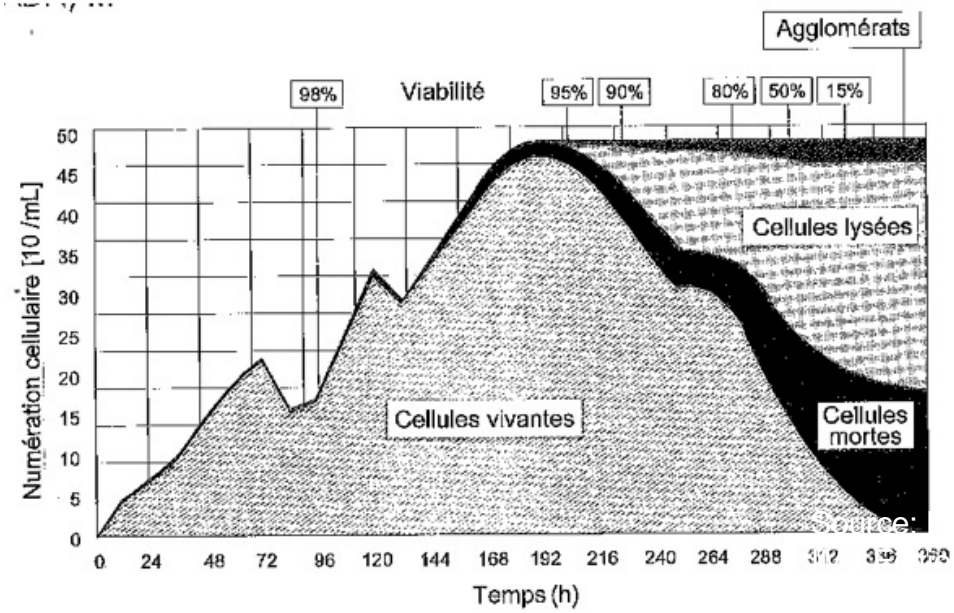


Full antibodies (mAb): 1200 aa
MM:150kDa, 1 glycosylation site

- ✅ Full antibodies and complex proteins
- ✅ Optimal glycosylation
- ✅ Full functional activity
- ✅ Optimized expression vectors

- ❌ Cost of the culture media
- ❌ Process lenght: cell line establishment, cell banks…
- ❌ Weak yields
- ❌ Viral security

Downstream process:What substances to eliminate during the purification steps?



- ✓ Host impurities:
  - Cell debris, nucleic acids, lipids
  - Host-cell proteins (HCP)
- ✓ Microorganisms:
  - Viral particles, Bactéria,
  - Pyrogen substances
- ✓ Process-related impurities:
  - Remaining buffer, chromatography résin, Métals, polymers «extractibles and leachables »…
- ✓ Product-related impurities:
  - Aggregated, truncated, unfolded…protein of interest forms

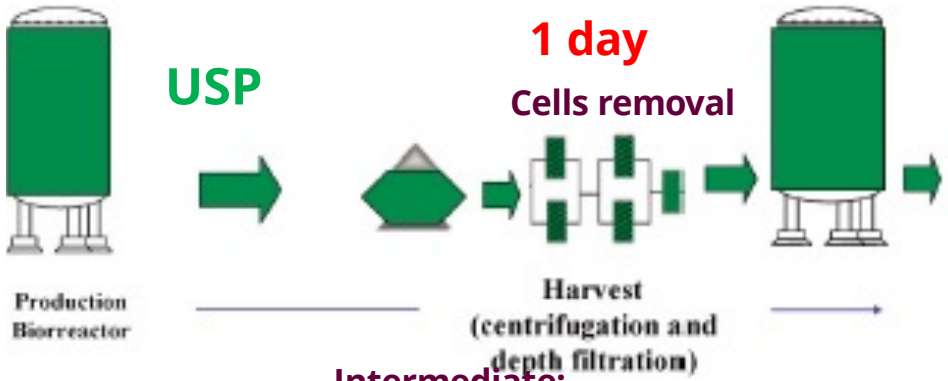
Industrial production of monoclonal antibodies mAbs

28 days

USP

1 day

Cells removal



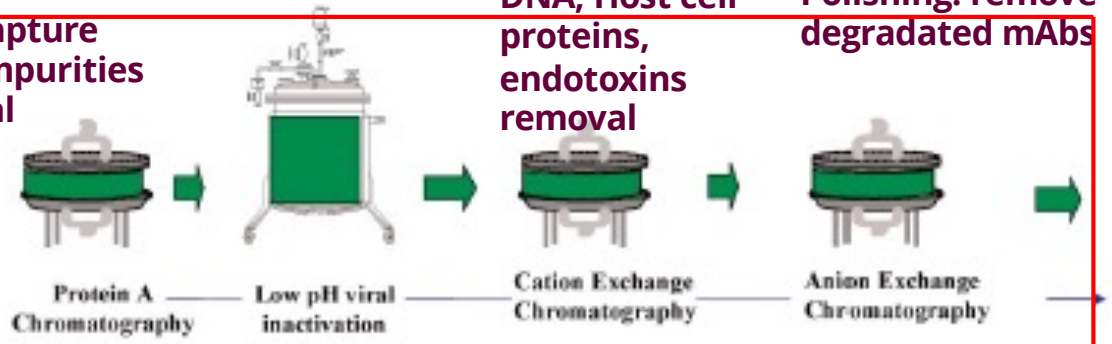
Centrifuge

DSP

mAb Capture
Most impurities removal

Intermediate: DNA, Host cell proteins, endotoxins removal

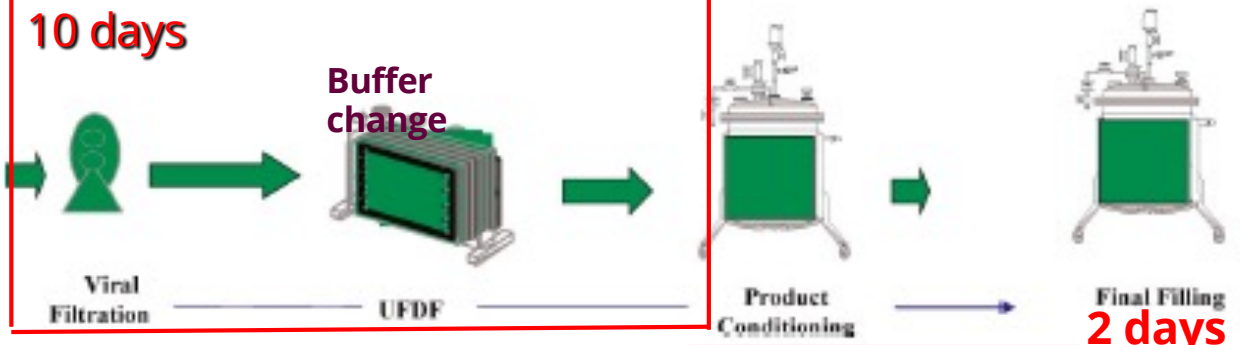
Polishing: remove degradated mAbs



Filtration

10 days

Buffer change



2 days

Chromatography

Product characterization 10 WEEKS

Yield 70-80%

Therapeutic protein characterization, A battery of validation tests

- **Identity / structure**

✓ Primary structure (AA composition)

✓ Secondary structure

✓ Glycosylation analysis

✓ Physical parameters: Mol weigth, isoelectic point…

- **Purity:**

✓ Host cell impurities (DNA, proteins, lipids…)

✓ Fabrication process imurities: leachates and extractables

✓ Product-related impurities: unfolded, truncated , aggregated, chemically degradated mAbs

- **Activity:**

✓ Target binding (affinity measurement)

✓ *In vitro* assays

✓ *In vivo* assays

- **Security:**

Virus particles or genome

Endotoxin detection

Which level?

EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

ICH harmonisation for better health

FDA U.S. FOOD & DRUG ADMINISTRATION

Conclusion

Pre-clinical trials

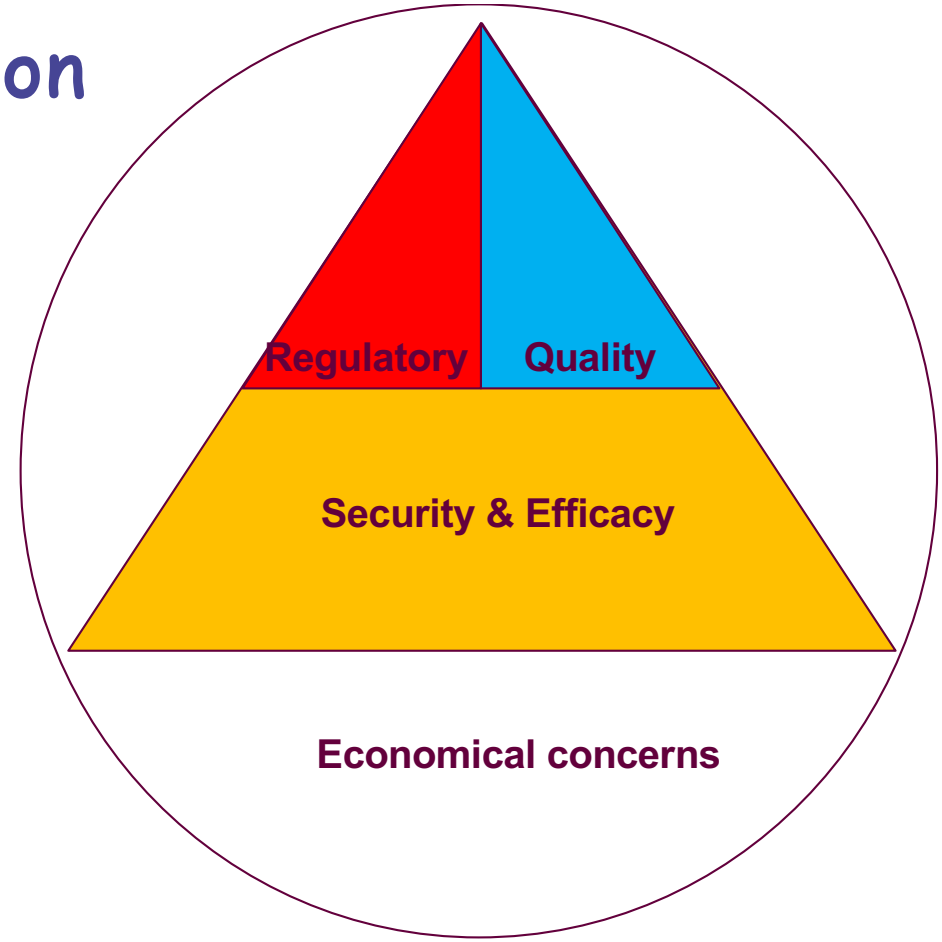
Clinical trials: I To IV

Good Laboratory Practice

Good Clinical Practice

Good Manufacturing Practice

« Good Distribution Practice »



Bioproduct production is under pharmaceutical regulatory concepts that are adapted to living material use

Define critical points at early development stages (MCB conception):
Process complexity
Quality controls
Equipment costs, Staff costs, time consuming

« DEVELOPING A PROCESS WITH THE END IN MIND »